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A qPCR MGB probe based eDNA assay for freshwater pearl mussel (*Margaritifera margaritifera* L.) in an Irish River

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Abstract 250 words

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Introduction max 2000 words

Freshwater pearl mussel *Margaritifera margaritifera* L, are large (~14 cm), long lived (>100 years) bivalves that are native to clean, fast-flowing, soft-water rivers and streams across Western and Northern Europe (Bauer 1986, 1992; Moorkens 1999; Young et al. 2001). Due to a degeneration habitat quality *M. Margaritifera* have

declined significantly across their range and are classified as critically endangered by the IUCN. Although large populations still exist, recruitment is low and the isolation of the subpopulations ensures low to no gene exchange occurs (Moorkens, 2011).

Within Western Europe, this has precipitated protection and conservation actions under national and international legislation including the listing of *M. margaritifera* under Annex II and V of the Habitats Directive (92:43: EEC). This has ensured that many rivers with *M. Margaritifera* sub populations have been designated as Special Areas of Conservation (SAC). The species is further protected under national legislation and is legally protected in Ireland under Schedule 1 of the Wildlife Act (Protection of Wild Animals) (Statutory Instrument No. 112, 1990) and the European Communities (Natural Habitats) Regulations (Statutory Instrument No. 94, 1997).

Although recent estimates have suggested a total 1282 sub populations, this is expected to be reduced to 204 sub populations by 2100. The island of Ireland currently supports 139 sub populations, with an expected number of subpopulations being reduced to 6 by 2100 (Mookhens et al., 2011). However, currently the species is still widespread in Ireland, although abundances have declined (Geist 2005). These subpopulations may provide a potential source for re-colonisation, given proposed improvements in future water and habitat quality-levels as outlined under the European Water Framework.

There is a recognised linkage between healthy stocks of salmonids such as brown trout (*Salmo trutta* L.) and Atlantic salmon (*S. salar* L.) and the endurance, growth and propagation and of *M. margaritifera* sub populations (Bauer 1979; Ziuganov and Nezlin 1988; Ziuganov *et al*. 1994). Studies have suggested a symbiotic releationship between salmonids and *M. margaritifera* - the mussels maintain water quality required by the salmonids and have been shown to reduce senility in salmon, thereby extending their life expectancy (Ziuganov 2005!). The salmonids gills host *M. margaritifera* glochidium, a larval stage of *M. margaritifera* that requires salmonids for dispersal. The chances of a glochidium successfully finding a host in waters with healthy salmonid stocks is as low as 0.0004%, with only 5% of these successfully attaching which can only survive for (Young and Williams 1984;). The pearl mussels have evolved to live in ultra- oligotrophic waters, where obligate salmonid host populations (e.g. brown trout) were never high and depend on the anadromous sea trout and salmon. Reductions in anadromous salmonid populations therefore have a drastic and immediate impact on

61 the successful attachment of glochidium and by extension recruitment and population viability of *M.*
62 *margaritifera* (Bauer 1979). Therefore, any recovery *M. margaritifera* populations is dependent on and
63 concomitant with the promotion of healthy salmonid populations. As an added value, the presence of *M.*
64 *margaritifera* beds aids the legal designation and maintenance of high quality salmonid nursery habitats for
65 through complementary legal protection.
66
67 *M. margaritifera* are important members of the food web in soft-water nutrient poor ecosystems; transferring
68 nutrients and energy from the water column to the sediments through filter feeding, stimulating production
69 across trophic levels (Spooner and Vaughn 2006). A previous study (Stoeckle et al. 2015) developed *M.*
70 *margaritifera* specific primers anchored in 16S mitochondrial (mt)DNA region and successfully deployed the
71 assay on environmental (e)DNA samples from Central Europe. However, while 16S sequences from different
72 organisms are abundant in public repositories, it would be advantageous to focus on the barcode of life gene
73 (mtDNA COI - http://www.barcodeoflife.org) as repositories of COI sequences contain representatives from
74 many more organisms than any other gene sequence repository. Further improvements of eDNA assays include
75 adding species specific probes with higher fidelity (e.g. minor grove binding, MGB probes) than both assays
76 based solely on species specific primers or those also incorporating TaqMan® probes not using the MGB group
77 (Kutyavin et al. 2000).
78
79 The aim of the current study was to develop an eDNA assay that can detect the presence and relative
80 abundances of *M. margaritifera* without hard sampling. This approach may allow for the detection of previously
81 unrecorded populations that would require recognition and a measure of protection. Remnant populations may
82 exist at densities too low for observation by traditional studies; acting as a potential source of recruits for
83 repopulation. The approach would also allow for the identification of *M. margaritifera* hotspots; zones that
84 support relatively a larger proportion of the mussel population.

85
86 **Methods**
87
88 *eDNA qPCR assay development*

All DNA tissue originated from a naturally diseased individual *M. margaritifera*. Found on the bank of the River Munster Blackwater (Fran Igoe personal comments). The tissue sample was received and total DNA was extracted from these tissue samples using the Qiagen Dneasy kit (Qiagen, Valencia, CA). Extracted DNA was used as template for assay validation and standard curves for qPCR. Species-specific primers for *M. margaritifera* (forward primer: 5′- TTG TTG ATT CGT GCT GAG TTA GG-3′, and reverse primer: 5′- GCA TGA GCC GTA ACA ATA ACA TTG-3′) and 5′-6-FAM labelled TaqMan® minor groove binding probe (5′-CCT GGT TCT TTG CTG GGT-3′) targeting region within the mtDNA cytochrome oxidase I (COI) region were designed using PRIMER EXPRESS 3.0 (Applied Biosystems-Roche, Branchburg, NJ). The total amplicon size, including primers, was 83 bp. Probe and primer sequences were matched against the National Centre for Biotechnology Information (NCBI - http://www.ncbi.nlm.nih.gov/) nucleotide database with BLASTn (Basic Local Alignment Search Tool) to confirm the species specificity for *M. margaritifera* in *in-silico* assays. The specificity and amplification capability of the assay was confirmed by conventional PCR amplification and DNA visualisation on a 1.5% agarose gel stained with SYBR® Safe - DNA Gel Stain (Life Technologies). In addition, to the qPCR eDNA assay for *M. margaritifera*, we included a previously developed eDNA qPCR assay (Gustavson et al. 2015) for brown trout (*S. trutta*) as a positive control for presence of amplifiable eDNA in water samples.

eDNA filtering and extraction of field samples

Water samples were collected from x locations were live *M. margaritifera* had been observed were in Munster Blackwater River in sterile 3 L PET bottles and kept frozen until analysed. Water samples were thawed in ambient temperature and 1 L per sample was filtered through individual 0.45 µm Whatman nitrate filters. The amount of water filtered was recorded for each water sample to the closest cL. Filters were subsequently dehydrated with 100% EtOH before storage at -20°C. Each filter was cut into halves (half for analysis and half for archival storage) and shredded to increase surface area for eDNA extraction using Qiagen QIAshredder (Qiagen, Valencia, CA). Total eDNA was extracted using a Qiagen DNeasy kit (Qiagen, Valencia, CA). Extracted eDNA was stored at -20˚C until further processing.

eDNA assay deployment

Concentrations of eDNA were determined by qPCR using an Applied Biosystems ViiA™ 7 (Life Technologies, Inc., Applied Biosystems, Foster City, CA) quantitative thermocycler. Amplification reactions for each species

included: 15 µl of TaqMan® Environmental Master Mix 2.0 (Life Technologies., Applied Biosystems, Foster City, CA), 3 µl of each primers (final concentration of 0.2 µM), probe (final concentration of 0.2 µM), ddH2O, and DNA template (3 µl), forming the 30µL reaction volume. The qPCR run method used warm-up conditions of 50˚C for 2 min and 95˚C for 10 min, followed by 40 cycles between 95˚C for 15 s and 60˚C for 1 min. The standard curve for *M. margaritifera* was generated with quantified (NanoDrop®-1000, Thermo Scientific, Wilmington, DE) DNA extractions from a tissue sample (DNA concentrations of 122.0 pg/L) using seven 10:1 serial dilutions as template for qPCR. The standard curve for *M. margaritifera* ($y = -3.4058x + 38.238$, $R^2 = 0.9997$, efficiency = 96.62%) was generated using 3 µl DNA template in a total reaction volume of 30 µl, respectively. The lowest concentrations of DNA (dynamic range) equalling 0.122 pg/L in the standard curves were detected at Cq (quantification cycle) 34.8 (average over three technical replicates, SD = 0.073). Results from the standard curve (Fig. 1.) indicate a dynamic range and lowest eDNA detection level at Cq 38.238 (equivalent to a *M. margaritifera* DNA concentration of 0.333 pg/L). All field samples were quantified in duplicates (two technical replicates), to ensure consistency, with two laboratory negative controls and *M. margaritifera*. Three water samples within the Munster Blackwater River system were used as template for *S. trutta* qPCR to validate the presence of amplifiable eDNA. The average Cq across technical replicates (n=2) were used for quantification.

Results and Discussion

The present study successfully developed an eDNA assay with very high sensitivity for *M. margaritifera*. All analysed samples yielded detectable eDNA (with Cq within the dynamic range) for both *M. margaritifera* and *S. trutta* (presence of *S. trutta* eDNA was validated in three sample locations), indicating that amplifiable target eDNA was present in all water samples. Resulting Cq values from the qPCR assays were transformed to pgDNA/L (based on the standard curve, Fig 1.). The concentrations of eDNA ranged from 0.462 pg/L in Rowls Aldworth West Bridge (right bank sample) to the highest of 109.884 pg/L in the Leader's Bridge Allow (left bank sample). Environmental DNA concentrations were relatively stable across transects within location (right bank, middle and left bank samples). Average eDNA concentrations (across transects) ranged from the lowest at in Rowls Aldworth West Bridge (1.056 pg/L) to the highest at Cullen Bridge (79.412 pg/L). Two graphs were plotted to visualise eDNA concentrations (Figs. 2 and 3). These results indicate variable eDNA concentrations among localities. The developed eDNA assay can be used to assess concentrations of eDNA which should be

Commented [JC1]: What do we do here. We do not need to have tons of locations. Just one field sample and one negative field sample…

related to the biomass of *M. margaritifera* and could be used for monitoring the status of *M. margaritifera* in individual locations and river systems. However, utilising the quantifying capabilities of eDNA assays requires careful planning, standardised and coordinated sampling efforts (exact GPS positions, dates, time of day, water levels, weather conditions, details about where in the water body samples were acquired from, etc.) to ensure that samples are of the highest quality. Nevertheless, the eDNA assay developed here can be used for rapid detection of *M. margaritifera* presence throughout Ireland and the natural range of *M. margaritifera*. Wide scale deployment of the assay can help detecting cryptic populations in watersheds where *M. margaritifera* has not previously been reported or where *M. margaritifera* are considered to have gone extinct.

This might aid and inform conservation efforts through the translocation of existing, although previously unreported, unviable subpopulations of *M. Margaritifera* in suboptimal habitats to either recently refurbished or pre-existing optimal habitats (clean water and salmonids). This will ensure gene transfer between sub populations and maintain genetic diversity in existing sub populations. The transferral of genetic material is pertinent given the additional stresses of climate change. Unfortunately, given the low levels of funding and political priority for conservation, this approach requires a hierarchical valuation of *M. margaritifera* habitats and the focusing of efforts on SAC designated viable habitats.

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173 **Conflict of Interest**: The authors declare that they have no conflict of interest.

174

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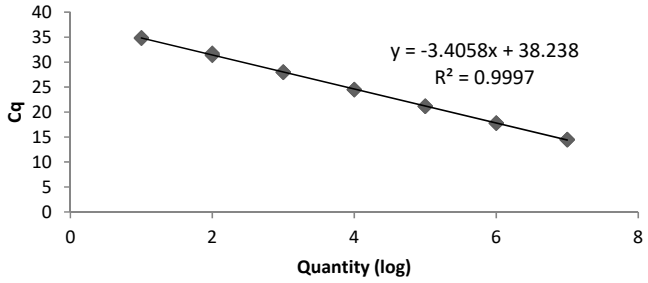

Fig. 1. Standard curve used to quantify Margaritifera margaritifera *eDNA concentrations. This curve is based on known concentrations and dilution series (10X) from a starting concentration of 0.122pg/L, Cq – quantification cycle.*

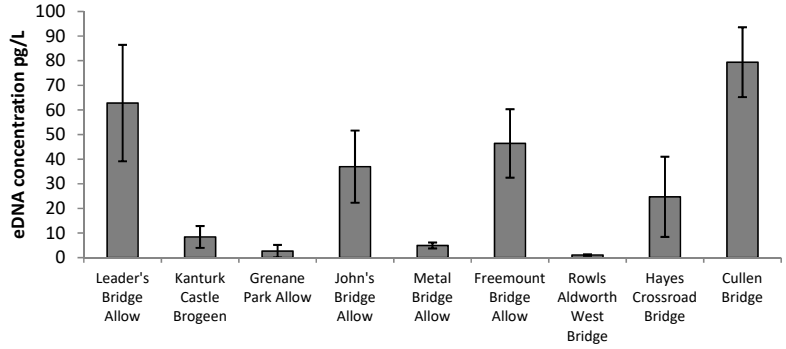

Fig. 2. Average concentrations (error bars indicate 95% C.I within locations) of Margaritifera margaritifera *eDNA among water samples from the Munster Blackwater river system.*

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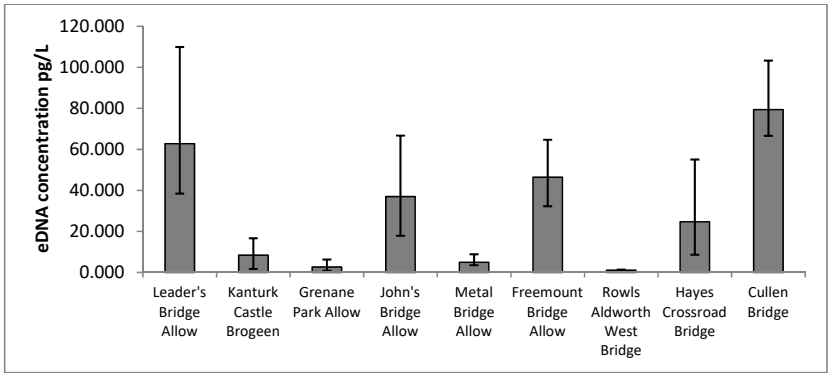

221 *Fig. 3. Average concentrations (error bars indicate observed max and min eDNA concentrations within*
222 *locations) of* Margaritifera margaritifera *eDNA among water samples from the Munster Blackwater river system.*
223

Appendix. Margaritifera margaritifera *eDNA concentrations within and among sites in the Munster Blackwater river system. (loc – location number, site L – left bank sample, site M - mid river sample, site R – right bank sample, Cq - quantification cycle, T1 – technical replicate 1, T2 – technical replicate 2).*

Location name	Loc	site	CqT1	CqT2	Average Cq	Average eDNA Conc across technical replicates pg/Ll	Average eDNA Conc across location pg/L
Leader's Bridge Allow	1	L	29.662	30.049	29.8555	96.410	62.796
		M	30.796	30.786	30.791	51.220	
		R	31.216	31.042	31.129	40.757	
Kanturk Castle Brogeen	2	L	35.053	34.082	34.5675	7.973	8.405
		M	36.844	35.107	35.9755	3.078	
		R	33.478	33.957	33.7175	14.165	
Grenane Park Allow	4	L	37.733	37.176	37.4545	1.132	2.660
		M	37.856	N/A	37.856	0.863	
		R	34.924	35.06	34.992	5.984	
John's Bridge Allow	7	L	32.672	33.373	33.0225	22.660	36.946
		M	32.806	32.355	32.5805	30.552	
		R	31.426	31.858	31.642	57.624	
Metal Bridge Allow	8	L	35.783	35.661	35.722	3.653	4.941
		M	34.412	35.289	34.8505	6.585	
		R	35.77	35.002	35.386	4.585	
Freemount Bridge Allow	9	R	31.772	31.471	31.6215	58.428	46.389
		M	32.498	32.361	32.4295	33.836	
		L	31.841	32.052	31.9465	46.903	
Rowls Aldworth West Bridge	12	L	N/A	N/A			1.056
		M	37.233	N/A	37.233	1.315	
		R	38.779	37.166	37.9725	0.798	
Hayes Crossroad Bridge	14	R	33.235	33.555	33.395	17.615	24.708
		M	34.458	34.378	34.418	8.821	
		L	32.133	31.711	31.922	47.686	
Cullen Bridge	16	R	30.779	30.881	30.83	99.775	79.412
		M	31.304	31.367	31.3355	70.892	
		L	31.385	31.428	31.4065	67.570	